Molecular detection of Sarcocystis lutrae in the European badger (Meles meles) in Scotland

Molecular detection of *Sarcocystis lutrae* in the European badger (*Meles meles*) in Scotland

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10 SUMMARY

11 Neck samples from 54 badgers and 32 tongue samples of the same badgers (*Meles meles*), collected
12 in the Lothians and Borders regions of Scotland, were tested using PCRs directed against the 18S
13 ribosomal DNA and the internal transcribed spacer (ITS1) region of protozoan parasites of the
14 family Sarcocystidae. Positive results were obtained from 36 / 54 (67%) neck and 24 / 32 (75%)
15 tongue samples using an 18S rDNA PCR. A 468 base pair consensus sequence that was generated
16 from the 18S rDNA PCR amplicons (KX229728) showed 100% identity to *Sarcocystis lutrae*. The
17 ITS1 PCR results revealed that 12 / 20 (60%) neck and 10 / 20 (50%) tongue samples were positive
18 for Sarcocystidae DNA. A 1074 bp consensus sequence was generated from the ITS1 PCR
19 amplicons (KX431307) and showed 100% identity to *S. lutrae*. Multiple sequence alignments and
20 phylogenetic analysis support the finding that the rDNA found in badgers is identical to that of *S.*
21 *lutrae.* This parasite has not been previously reported in badgers or in the UK. *Sarcocystis lutrae*
22 has previously only been detected in tongue, skeletal muscle and diaphragm samples of the
23 Eurasian otter (*Lutra lutra*) in Norway and potentially in the arctic fox (*Vulpes lagopus*).

24

25 Keywords

26 *Sarcocystis lutrae*, rDNA, Scotland, European Badgers (*Meles meles*), 18S PCR, ITS1 PCR,
27 Phylogenetic analysis

KEY FINDINGS

- A 468 bp consensus sequence generated from the 18S rDNA of badger samples showed 100% identity to the 18S sequence for *Sarcocystis lutrae.*
- A 1074 bp consensus sequence generated from the ITS1 region of badger samples showed 100% identity to the ITS1 sequence of *S. lutrae*.
- 40 / 54 (74%) of badgers had at least one positive sample for *S. lutrae* DNA.
- This is the first reported detection of *S. lutrae* DNA (18S rDNA and ITS1 region) in badgers in the United Kingdom.

INTRODUCTION

Current knowledge suggests that Sarcocystosis is caused by 200 currently identified species of single-cell coccidian parasites in the phylum Apicomplexa, and genus *Sarcocystis*. These parasites infect a wide range of definitive and intermediate hosts, including carnivorous animals, domestic animals and humans (Dubey *et al.*, 2015; Dubey & Lindsay, 2006; Kaltungo & Musa, 2013). All *Sarcocystis* parasites have an obligatory two host life cycle (some exception such as *S. neurona* exist); asexual reproduction takes place in the intermediate host and sexual reproduction occurs in the intestine of the definitive host (Dubey & Lindsay, 2006). *Sarcocystis neurona* has a very broad intermediate host range and for example sporocysts from opossums can infect many hosts, of which some are natural intermediate hosts (in which sarcocysts are formed), while others are aberrant hosts (in which only schizonts are formed) (Dubey *et al.*, 2001a). Transmission from definitive to intermediate host occur via the ingestion of oocysts/sporocysts from faeces via contaminated food or water, and transmission from intermediate to definitive host occur via the ingestion of sarcocysts which are found in muscle tissue (Dubey & Lindsay, 2006; Gjerde & Josefsen, 2015). However, knowledge and understanding of all the life cycle stages of *Sarcocystis* species in wild carnivores is incomplete and needs to be researched in more detail to help us to better understand disease pathogenesis, symptomatology and impact of parasite diversity. More research is needed to determine the range of clinical manifestations of *Sarcocystis* infections in wild carnivores, as there is little information available about the signs and symptoms in these host species.

Only a few *Sarcocystis* species have been identified in wild carnivores of the family Mustelidae (Dubey *et al.*, 2010). For example *S. neurona* has previously been identified in (Eurasian) otters (*Enhydra lutris*) (Dubey *et al.*, 2003; Dubey *et al.*, 2001b; Miller *et al.*, 2009; Wendte *et al.*, 2010), while *S. lutrae* has been found in the Eurasian otter (*Lutra lutra*) in Norway (Gjerde & Josefsen, 2015). *Sarcocystis lutrae* has not been confirmed in another host species other than the Eurasian otter and potentially arctic foxes (Gjerde & Josefsen, 2015; Gjerde & Schulze, 2014). Various *Sarcocystis* spp. including *S. hofmanni, S. melis, S. cf. sebeki* and *S. cf. gracilis*,

have previously been recorded by light microscopy (LM) and transmission electron microscopy (TEM) in heart, thigh, loin, thorax and tongue samples in European badgers (*Meles meles*) from Berlin (Odening *et al.*, 1994a; Odening *et al.*, 1994b). None of these *Sarcocystis* species in badgers have been identified in the United Kingdom. More recently, an unnamed species of *Sarcocystis* was recorded in the tongue, diaphragm, and masseter muscle of Japanese badgers (M*eles anakuma*) using haematoxylin and eosin (H&E) staining (Kubo *et al.*, 2009). To date LM of fresh muscle tissue and TEM have been examined to identify the *Sarcocystis* spp. found in badgers, meaning no DNA sequences are available for the *Sarcocystis* species previously identified in badgers. Only a few *Sarcocystis* species found in wild carnivores have been examined using molecular methods these include species, such as *S. arctica*, *S. lutrae, S. kalvikus, and S. kitikmeotensis (Dubey et al., 2015).* Techniques, such as PCR and sequence analysis are more frequently used to identify *Sarcocystis* species (Gjerde & Josefsen, 2015). Polymorphisms in the 18S rDNA and ITS1 region may help with the speciation and discrimination of the different species within the *Sarcocystis* genus. The aim of this study was to determine the prevalence and species of *Sarcocystis* in muscle samples from European badgers (*Meles meles*).

MATERIALS AND METHODS

Collection of samples

Fifty-four European badger (*M. meles*) carcasses were collected from around the Lothians and Borders regions of Scotland, following fatal collisions with vehicles (badgers were collected with the knowledge and permission of Scottish Natural Heritage) (Bartley *et al.*, 2013). Carcasses were stored at -20°C prior to processing, full necropsies were performed when possible where samples of neck muscle, tongue, spleen, submandibular lymph node, liver, lung, brain, heart, blood and spinal cord were collected.

87 *DNA extraction*

88 DNA was extracted from muscle samples of 54 badgers. From those badgers, 54 neck samples and
89 32 tongue samples derived from the same animals were extracted. Approximately 1g of each
90 thawed tissue was transferred into a separate CK22 Precellys tissue homogenizer tube (Cepheid,
91 Stretton Derbyshire, UK), containing 1ml Nuclei Lysis Solution (Promega, Madison, WI, USA).
92 Samples were homogenized for 2 x 50s at 6500rpm using a Precellys 24 tissue homogenizer
93 (Depheid, Stretton Derbyshire, UK). 400µl of each homogenised tissues were added to a further
94 900µl of nuclei lysis solution and incubated at 55^{o}C overnight. Samples were then processed using
95 the Wizard® genomic DNA (Promega, Madison WI, USA) purification protocol, which was adapted
96 to use 0.4g of starting material (Bartley *et al.*, 2013).
97
98 *Detection of protozoan DNA by 18S PCR and ITS1 PCR*

99 Parasite DNA was detected using a nested PCR, targeting the multi-copy 18S rDNA of the
100 ribosomal RNA gene family. The first round PCR used external primers that recognised various
101 apicomplexan parasites including *Neospora caninum*, *Toxoplasma gondii* and *Sarcocystis spp*
102 (Supplemental Table 1). Briefly, each 20µl reaction contained 2µl of 10x custom PCR mix- (45mM
103 Tris-HCl, 11mM $(NH_4)_2SO_4$, 4.5mM $MgCl_2$, 0.113mg/ml BSA, 4.4µM EDTA and 1.0mM each of
104 dATP, dCTP, dGTP and dTTP) (ABgene, Epsom, Surrey, UK), 0.25pM of each primer (Eurofins
105 MWG Operon), 0.75 units of BioTaq (Bioline, London, UK), 13.85µl of water and 2µl of sample
106 DNA (Burrells *et al.*, 2016). The PCR conditions for the first round were 95°C for 5 mins followed
107 by 35 cycles at 95°C for 1 min, 56°C for 1 min and 72°C for 1 min, with the final extension period
108 at 72°C for 5 mins. The primary PCR amplicons were diluted with 100µl DNase/RNase free water
109 and 2µl of the diluted primary amplification product was added as template DNA for the second
110 round amplification. Second round primers were designed to amplify only *Sarcocystis* spp.
111 (Supplemental Table 1). The specificity of the primers were tested using *S. neurona, S. lutrae, S.*

gigantea, S. tenella, S. rileyi, S. fayeri, N. caninum and T. gondii DNA samples (Data not shown). The reaction conditions for the second round PCR were identical to the first round, with the exception that internal forward and reverse primers were used. Each batch of samples analysed contained a positive control: *S. lutrae* (obtained from this study) and negative controls: *N. caninum, T. gondii* and water and were tested in duplicates. With each batch of badger samples extracted, a negative (water) 'extraction control' was tested (Bartley *et al.*, 2013). Badgers that showed strong positive bands for both neck and tongue samples in the 18S rDNA PCR, were tested further using the ITS1 PCR. Here, Sarcocystidae were detected using the primers 'SU1F' and '5.8SR2' that amplify the ITS1 region (~1000 bp) and targets the adjacent 18S and 5.8S rDNA genes, respectively (Gjerde, 2014) (Supplemental Table 1). The reaction conditions for the ITS1 PCR were identical to those of the 18S rDNA PCR. The ITS1 region was selected to differentiate members of the group Sarcocystidae as it is highly polymorphic compared to the 18S rDNA. PCR products (6µl) were analysed by 2% agarose gel electrophoresis, stained with gel red (1:10,000) (Biotonium, Hayward, USA) and visualised using UV light. Each batch of samples analysed by ITS1 PCR contained a positive control: *S. lutrae* and negative control water.

Cloning, DNA sequencing and sequence assembly

The PCR products from twelve animals (both positives for tongue (n = 12) and neck muscle (n = 12)) using the 18S external primers and six positive PCR products from four animals (positives tongue (n =4) and neck (n = 2)) using the external ITS1 PCR, were purified using the commercially available Wizard® SV Gel and PCR Clean-up System (Promega, Madison WI, USA). The PCR products were eluted in 50µl of DNase / RNase free water and the nucleic acid concentration was determined by spectrophotometer (Nanodrop, ND1000). For each sample, 100ng of DNA was sent for sequencing (Eurofins MWG Operon). The 18S amplicons were sequenced with the 18S primers and the ITS1 amplicons were sequenced with the ITS1 primers (Supplemental Table 1).

Three first round PCR amplicons from the 18S rDNA PCR (tongue n= 1, neck n = 2) and a further 3 PCR amplicons from the ITS1 PCR (tongue n= 2, neck n = 1) were cloned using the pGEM®-T Easy Vector System (Promega, Madison WI, USA) as previously described (Bartley *et al.*, 2016) with the following alterations. Two microliter (64ng) of the purified product were ligated into the pGEM®-T Easy Vector (1µl at 50ng/µl) (Promega, Madison WI, USA) according to the manufacturer's instructions. Following ligation, 1µl (8ng) of ligated vector/insert was used to transform 40µl of high-efficiency competent JM109 cells (≥1 × 10^8cfu/µg DNA) (Promega, Madison WI, USA) using manufacturer's instructions. A successful transformation was confirmed using LB agar plates containing 100µg/ml ampicillin, spread with 100µl of IPTG (Isopropyl β-D-1-thiogalactopyranoside) (100mM) and 20µl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (50mg/ml). White colonies were screened by PCR using the 18S external primers and the SU1F and 5.8SR2 primers to confirm the presence of the *Sarcocystis* 18S rDNA and ITS1 region insert. Three clones from each of the three badger samples from each of the 18S (n = 9 clones) and ITS1 (n = 9 clones) PCR were sequenced (Eurofins, MWG Operon) using T7 and SP6 primers. Additional internal S-ITS1-F and S-ITS1-R primers (Supplemental Table 1) were used for the ITS1 clones to ensure a double stranded consensus sequence of over 1000 bp was generated. Overall consensus sequences were generated for the 18S and ITS1 amplicons from each badger.

A Basic Local Alignment Search Tool (BLAST) search was completed to determine percentage identity of the generated sequences against previously published sequences. Multiple sequence alignments were performed using the BioEdit sequence alignment editor 7.1.3.0. to show the difference between the closely related *Sarcocystis spp* . Phylogenetic analyses were performed on both the 18S rDNA and ITS1 consensus sequences using MEGA6 software (Tamura *et al.*, 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood. The tree is drawn to scale, with branch lengths measured in the

number of substitutions per site and all positions containing gaps and missing data were eliminated. The phylogeny was tested with the bootstrap method, using 1000bootstrap replications.

Statistical analysis

The proportion of positive samples (prevalence), with confidence intervals (95% CI) was calculated for the presence of *Sarcocystis* DNA in the tongue and neck muscle samples from badgers. The numbers of badgers where either tongue, or neck muscle sample were positive and those animals where both samples were positive were also calculated. All of the calculations were carried out using the Minitab 17 software (v17.1.0.0).

RESULTS

Screening of samples for the presence of protozoan DNA using the 18S rDNA PCR

Five badgers were initially screened and DNA samples from leg, neck muscle, tongue, sub-mandibular lymph node, liver, lung, brain, heart and spleen, were tested using the 18S external primers in a single round PCR. Positive PCR amplicons were observed for 1/5 leg muscle, brain and lung sample, 2/5 neck muscle samples and 3/5 tongue and spinal cord samples. Sequencing PCR amplicons from one neck muscle and two tongue samples showed identity to *S. lutrae* (accession KM657770). The PCR products from tongue and neck were the only samples to produce identifiable sequences, and thus these organs were selected for further testing. Spinal cord was not selected for further analysis, due to the limited numbers of samples available (n= 12).

Verification of PCR specificity and sequencing

The *Sarcocystis* specific 18S rDNA nested PCR was used to screen all muscle samples available, tongue (n=32) and neck muscle samples (n=54) from 54 badgers. The results showed that 36 / 54 (67%) (95% CI: 52.5%, 78.9%) neck samples and 24 / 32 (75%) (95% CI: 56.5%, 88.5%) tongue

188 samples tested positive for *Sarcocystis* DNA. Twenty badger samples showed positive PCR results
189 for *Sarcocystis* DNA in both neck and tongue samples using the 18S rDNA PCR (20 / 32) (95% CI:
190 43.6%, 78.9%). Forty badgers tested positive with the *Sarcocystis* specific 18S rDNA PCR with at
191 least one tissue (40 / 54, 74%) (95% CI: 60.3%, 85.0%). No amplified products were observed for
192 the negative controls: water, *T. gondii* and *N. caninum*. Badgers (n=20) that showed positive results
193 for both tongue and neck in the 18S rDNA PCR were tested using the ITS1 PCR (Gjerde, 2014).
194 The ITS1 PCR revealed positive results for 12 / 20 (60%) (95% CI: 36.0%, 80.8%) neck and 10 / 20
195 (50%) (95% CI: 27.1%, 72.8%) tongue samples for Sarcocystidae DNA. No PCR amplicons were
196 generated for 4 / 20 badgers tested using the ITS1 primers.

197 Consensus sequences were generated for the 18S rDNA from 9 clones: tongue (n=3) and
198 neck (n=6) and for the ITS1 region from 9 clones: neck (n=3); and tongue (n=6). These clones were
199 used to create consensus sequences for the 18S rDNA and ITS1 amplicons for each animal. The 3
200 consensus sequences, each for the 18S rDNA and the ITS1 amplicons, were identical to each other
201 and were used to create a general consensus sequence for both the 18S rDNA and ITS1 regions. The
202 general consensus sequences for the 18S rDNA (468 bp) and the ITS1 region (1074 bp) were
203 submitted to Genbank (KX229728 and KX431307, respectively). When the 18S rDNA
204 (KX229728) and ITS1 (KX431307) sequences generated during this study, were compared on
205 NCBI BLAST against published DNA sequences, it was found that the 18S rDNA fragments
206 showed 100% identity to isolates of *S. lutrae* (18S rDNA: KM657770). The ITS1 sequence showed
207 99.2-100% identity to the 22 ITS1 sequences of *S. lutrae* found in the Eurasian otter (*Lutra lutra*)
208 (Gjerde & Josefsen, 2015).

209

210 *Phylogenetic relationship and multiple sequence alignments of S. lutrae and related species*

211 Phylogenetic analysis revealed that the *S. lutrae* rDNA found in badgers appears in the same clade
212 as the *S. lutrae* found in otters, as well as the closely related species *S. rileyi* and *S. turdusi* (Figure 1
213 A). The multiple sequence comparison demonstrated that the 18S rDNA fragment found in badgers

(KX229728) is identical to *S. lutrae* found in otters (KM657775) (Figure 2 A). The sequence alignment of the 18S rDNA (Figure 2 A) shows polymorphic and conserved regions for the closely related *Sarcocystis* species. The alignment shows that our sequence and *S. lutrae* are identical to each other but are distinct from the other closely related species sequences by one additional 'T' base in comparison to *S. corvusi* and *S. arctica* and *S. turdusi,* and multiple base pair differences from *S. rileyi, S. lacerate, S. mucosa and S. neurona* (Figure 2 A). The ITS1 region was also used for sequence alignments and phylogenetic analysis, since the 18S rDNA gave poor discrimination of closely related species. The ITS1 phylogenetic analysis showed a clearer differentiation from the closely related *Sarcocystis spp* (*S. corvusi, S. arctica, S. neurona, S. turdusi* and *S. rileyi*), however *S. kalvikus* was found in the same clade as *S. lutrae* from badgers and *S. lutrae* from otters (Figure 1 B). Yet, when using the ITS1 multiple sequence analysis, it can be seen that *S. kalvikus* can be distinguished from the *S. lutrae* found in badgers and *S. lutrae* found in otters. The ITS1 region is more polymorphic compared to the 18S rDNA and the ITS1 sequence comparison showed a clear differentiation of *S. lutrae, S. kalvikus*, *S. turdusi, S. corvusi* and *S. arctica* (Figure 2 B). From the multiple sequence analysis of both the 18S rDNA and ITS1 regions, it can be clearly seen that the *Sarcocystis spp* rDNA fragments found in the sample of badgers in this study are identical to the *S. lutrae* found in otters.

DISCUSSION

In this paper we report the detection of 18S rDNA and ITS1 region in tongue and neck muscles of European badgers (*M. meles*) collected from around the Lothians and Borders regions of Scotland, that show 100% sequence identity to DNA from *S. lutrae* (KM657770 and KM657775). *Sarcocystis lutrae* has previously been identified in tongue, skeletal muscle and diaphragm in the Eurasian otter (*Lutra lutra*) (Gjerde & Josefsen, 2015). Moreover, Gjerde & Schulze (2014), found that the cox1 sequence of *S. lutrae* from otters were identical with one of the cox1 sequence from an arctic fox

harbouring *S. arctica*. One of those cox1 sequences was initially assigned to *S. arctica* even though it differed slightly from the other cox1 sequences obtained and was later re-assigned to *S. lutrae* and thus the presence of *S. lutrae* in Arctic foxes can be disputed (Gjerde & Josefsen, 2015). This study used the 18S rDNA and ITS1 region, to verify and identify the 18S rDNA fragments found in badger samples. Phylogenetic and multiple sequence alignments have shown that the ITS1 region is more polymorphic, compared to the 18S rDNA gene. Using multiple loci, such as the 18S rDNA gene and the ITS1 region will help species identification and is more reliable than using one locus alone. Few polymorphic regions in the 18S rDNA gene have previously been shown in especially closely related *Sarcocystis* species (i.e. *S. lutrae, S. turdusi, S. arctica, S. wobeseri*) and little sequence data has been generated. It has been shown that the ITS1 region gives a clearer differentiation for these species (Gjerde & Josefsen, 2015; Gjerde & Schulze, 2014). Since the ITS1 region is not a gene, higher mutation densities are tolerated, making this region highly variable among species and thus a useful marker for species identification for some, however, not all *Sarcocystis* spp.

From this study it can be confirmed that the 18S rDNA and ITS1 region identified in badgers showed 100% sequence identity to *Sarcocystis lutrae* (KM657770 and KM657775), indicating that the *Sarcocystis* species detected is likely to be *S. lutrae*. Active infections may have been detected if LM of fresh muscle tissue, such as tongue muscle, were analysed. *Sarcocystis hofmanni, S. melis, S. cf. sebeki, S. cf. gracilis* and an unnamed *Sarcocystis* species have previously been recorded in European badgers (*M. meles*) and Japanese badgers (*M. anakuma*) using TEM and, LM of fresh muscle tissue (Kubo *et al.*, 2009; Odening *et al.*, 1994a; Odening *et al.*, 1994b). Since those studies were conducted before molecular techniques were used in such research, no 18S or ITS1 sequences were generated for these species. Testing both neck muscle and tongue for the detection of *Sarcocystis* DNA proved advisable, as both these tissues showed a high presence of *Sarcocystis* DNA and if only one tissue was tested the overall prevalence would have been lower. The density of sarcocysts may vary in different types of muscle tissues, such as the diaphragm,

oesophagus, tongue and heart. These tissues are commonly used to demonstrate the presence of sarcocysts in hosts (Dubey *et al.*, 2015).

Identification of *Sarcocystis* species based on morphology employs looking at structural characteristics, such as sarcocyst wall and morphology; however, more than one *Sarcocystis spp* may have the same sarcocysts morphology and the same species can occur in different hosts (Dubey *et al.*, 2015; Dubey *et al.*, 1989). More recently molecular methods of *Sarcocystis* spp. have been particular useful to distinguish between morphologically indistinguishable species in closely related intermediate hosts, such as water buffaloes and cattle, and different cervids. Ideally, individual sarcocysts should be excised from fresh muscle tissue, examined in wet mounts by LM, used to extract DNA for molecular characterisation and fixed to study them using TEM. Using both identification methods would allow phenotypic and genotypic data to be combined and be linked to the species description. Using morphological characteristics alone for the identification may prove difficult as size and shape is subject to change depending on the age of the Sarcocysts but this observation can be strengthened by sequencing DNA amplicons from different regions of the parasite genome (Dubey *et al.*, 2015).

The data presented in this study shows that the DNA detected in European badgers showed sequence identity at two different loci to *S. lutrae* found in otters. This shows that badgers from the Lothians and Borders regions of Scotland are frequently infected with *S. lutrae*. Badgers are omnivores, and it is likely that they become infected through the ingestion of sporocysts shed by definitive host (predator/scavenger) (Dubey & Lindsay, 2006). Birds, such as the white-tailed (sea) eagle (*Haliaeetus albicilla*) suggested by (Gjerde & Josefsen, 2015) or birds of the family *Corvidae*, as well as other badgers and foxes (*Vulpes vulpes*) may act as a definitive host of *S. lutrae*. Further research, involving microscopic analysis, as well as multiple locus sequence typing, is needed to confirm whether *S. lutrae* is widely distributed across Great Britain and whether *S. lutrae* is only found in badgers, (Eurasian) otters and potentially arctic foxes.

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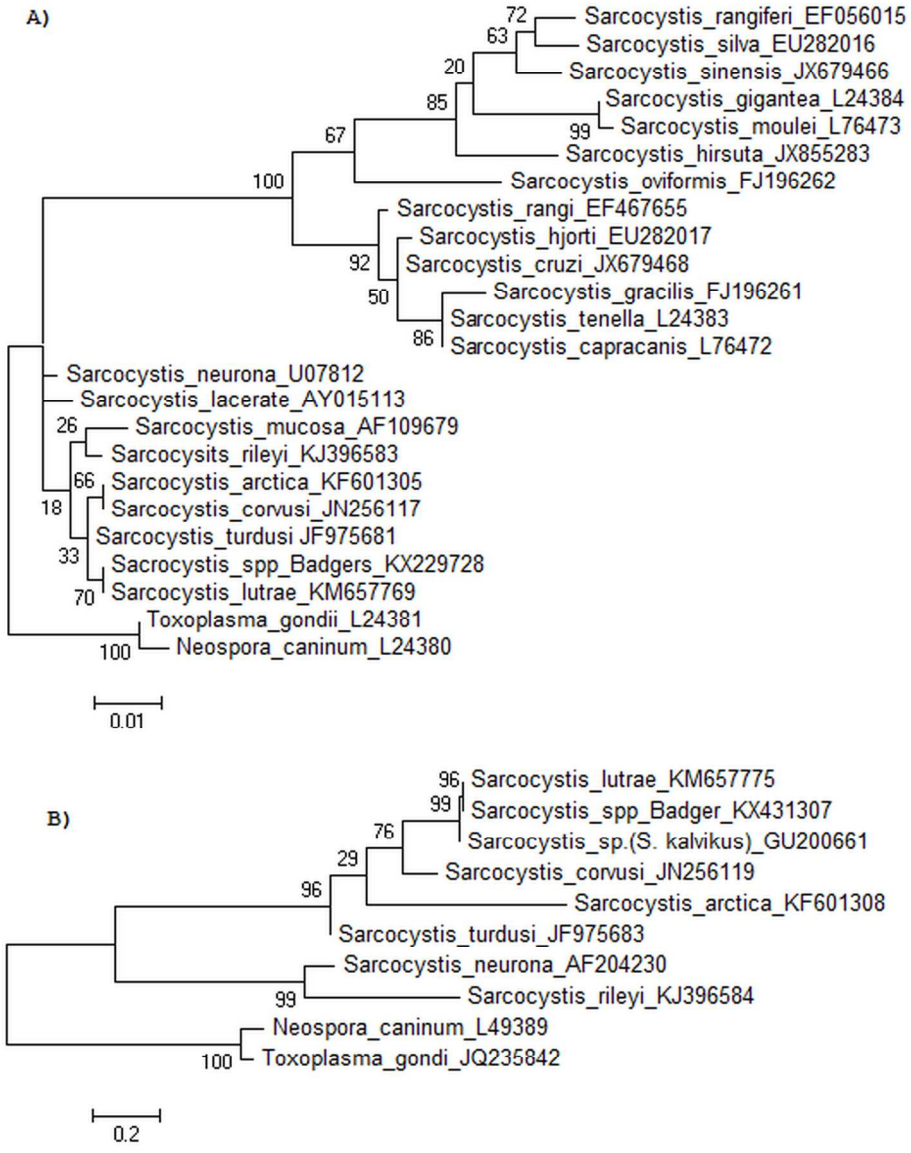
Table 1. Sequences and specificity of primers used for the detection of Sarcocystis spp DNA in badger samples.

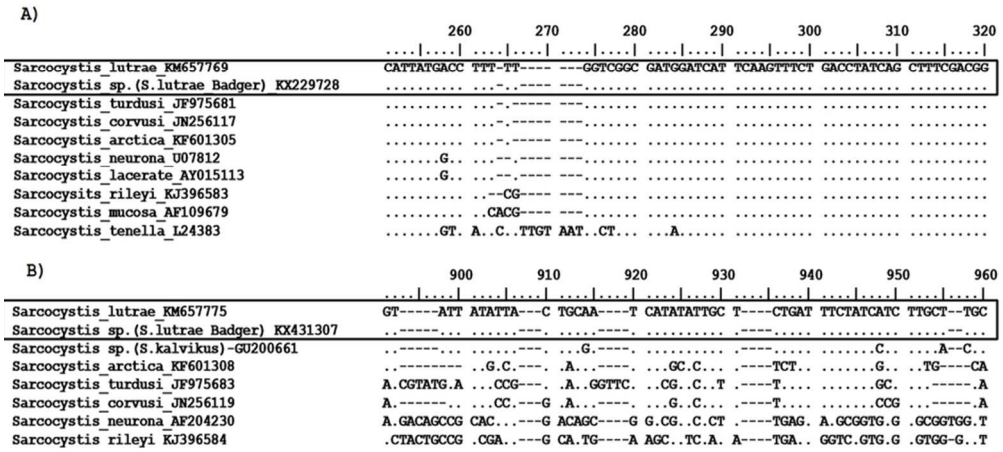
Region		Primer	Froward Primer		Species amplified	Reference
18S	External	Forward	NTS-18S-F1	5′-GCC ATG CAT GTC TAA GTA TAA G -′3	*N. caninum, T. gondii* and *Sarcocystis spp.*	This study
		Reverse	NTS-18S-R1	5′-CCT ATC ATT CCA ATC ACT AGA AAT -′3		
	Internal	Forward	NTS-18S-F2	5′- GGA TAA CCG TGG TAA TTC TAT G -′3	*S. lutrae, S. neurona* and *S. lacertae*	
		Reverse	S-18S-G9	5′- CAT CGC CGA CCA AAA AGG -′3		
ITS1	External	Forward	SU1F	5′- GAT TGA GTG TTC CGG TGA ATT ATT -′3	*Sarcocystidae*	Gjerde, 2014
		Reverse	5.8SR2	5′- AAG GTG CCA TTT GCG TTC AGA A -′3		
	Internal	Forward	S-ITS1-F	5′- TTT CTG TAG CGT TGA GAG GAG T -′3	*Sarcocystidae*	This study
		Reverse	S-ITS1-R	5′- CGC CTC GCT CAA CAT CAT CAT AAA -′3		

Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method for selected members of the Sarcocystidae. A) 18S rDNA with the highest log likelihood (-1280.9024) and B) ITS1 spacer region with the highest log likelihood (-2396.9991). The percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Figure 2. Multiple sequence alignment of the polymorphic sections of the 18S and the ITS1 region amplified in this study: A) 18S rRNA gene region, B) ITS1 region. Boxing shows identical sequences of S. lutrae (18S KM657769 and ITS1 KM657775) and the Sarcocystis sequence detected in the badger samples (18S KX229728 and ITS1 KX431307). Dots represent identical base pairs and dashed lines represent gaps in the alignments. Numbers given above each alignment correspond to the nucleotide position in the sequence A) KM657769 and B) KM657775, respectively.



203x248mm (300 x 300 DPI)



80x38mm (300 x 300 DPI)